Biochemical Pharmacology, Vol. 16, pp. 2387–2393. Pergamon Press Ltd. 1967. Printed in Great Britain

THE EFFECT OF 2,2′-DITHIODIPYRIDINE ON THIOLS AND OXIDIZABLE SUBSTRATES OF EHRLICH ASCITES CELLS AND OF NORMAL MOUSE TISSUES*

D. R. GRASSETTI and J. F. MURRAY, JR.

Arequipa Foundation, Russ Building, San Francisco, Calif., U.S.A.

(*Received* 3 *May* 1967; *accepted* 28 *June* 1967)

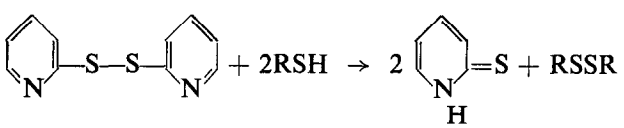
Abstract—(1) 2,2′-Dithiodipyridine (2-PDS) reacts with thiols with formation of a disulfide and 2-thiopyridone. The latter compound can be determined by its u.v. absorption. The inhibition of respiration of Ehrlich ascites cells by 2-PDS is probably due in large extent to the oxidation of cell thiols to disulfides. When enzymatic reactions do not occur, as in heat-denatured ascites cells and mouse tissues, a smaller amount of 2-PDS is consumed than with live tissues.

(2) The oxidation of glutathione by 2-PDS (GSH → GSSG) can be coupled to the reduction of GSSG by NADPH (catalyzed by glutathione reductase). This leads to continuous reoxidation of the GSH formed, and the overall process is the oxidation of NADPH by 2-PDS (a reaction that does not occur spontaneously).

(3) 2-PDS is a substrate for glutathione reductase, oxidizing NADPH at about 1/200 the rate of glutathione. This reaction can be coupled to the oxidation of glucose 6-phosphate by $NADP^+$ (catalyzed by glucose 6-phosphate dehydrogenase).

(4) The reactions described in (2) and (3) above could take place in the cell, and thus account for the "extra" amount of 2-PDS used by metabolically active cells.

WE HAVE recently shown[1] that 2,2′-dithiodipyridine† (I) reacts with thiols, with formation of 2-thiopyridone (II) and a disulfide (Reaction 1). This reaction, and the analogous reaction of 4,4′-dithiodipyridine, are the basis of a method for the determination of sulfhydryl groups in biological materials.[1]



(I) (II) (1)

During the course of that work, it was found that live cells or tissue slices consume relatively larger amounts of 2-PDS that the corresponding heat-denatured tissues. We decided to investigate this finding, in order to determine the cause of the discrepancy. It is shown in the present communication that the consumption of "extra"

* This investigation was supported by Public Health Service Grants CA 10065 and CA 08538 from the National Cancer Institute, National Institutes of Health, Bethesda, Md.

† Abbreviations used in this paper: 2-PDS = 2,2′-dithiodipyridine; 2-TP = 2-thiopyridone; $NADP^+$, NADPH = oxidized and reduced forms of nicotinamide adenine dinucleotide phosphate; NAD^+, NADH = oxidized and reduced forms of nicotinamide adenine dinucleotide; GSH, GSSG = reduced and oxidized forms of glutathione; GR = glutathione reductase [NADPH, (NADH): GSSG oxidoreductase, EC 1.6.4.2]; KRP buffer = Krebs–Ringer phosphate buffer at pH 7·2[2]; G6P-DH = glucose 6-phosphate dehydrogenase (D-glucose 6-phosphate:$NADP^+$ oxidoreductase, EC 1.1.1.49).

amounts of 2-PDS by metabolically active tissues could be due to the enzyme-mediated oxidation of cofactors (e.g. NADPH and NADH) and metabolites (e.g. glucose 6-phosphate) by 2-PDS.

EXPERIMENTAL

2,2'-Dithiodipyridine was prepared according to Marckwald *et al.*[3] and used as the free base. The purity of this compound was established by thin-layer chromatography as described before.[1] Glutathione reductase (yeast), glucose 6-phosphate dehydrogenase (yeast), NADPH, NADH, and glucose 6-phosphate were purchased from Sigma Chemical Co.

Sulfhydryl determination. Ehrlich ascites fluid was harvested from 7- to 10-day transplants in female Swiss mice; the cells were separated by centrifugation at low speed (10-12 *g* for 10 min), resuspended in KRP buffer and centrifuged again. Two to three washings with KRP buffer were usually sufficient to remove any red blood cells. The ascites cells were resuspended in a sufficient volume of KRP buffer to yield 100×10^6 cells/ml. This suspension (0·1 ml, 10×10^6 cells) was incubated in stoppered flasks with 4·9 ml of 10^{-3} M 2-PDS in KRP buffer, for 1 hr at 37° with mechanical agitation. At the end of this period, the mixture was filtered through Whatman glass fiber filter paper GF/C and the 2-TP present in the filtrate was determined by its absorption at 343 mμ, as described before.[1]

Liver and kidney were excised from normal female Swiss mice. After washing with buffer, slices (0·5 mm think) of these tissues were prepared with a McIlwain–Buddle tissue chopper.[4] The slices (60–80 mg) were suspended in 5 ml KRP buffer containing 4·0 μmole 2-PDS and incubated for 1 hr at 37° in a metabolic shaking bath. The slices were then separated by filtration through glass fiber paper and 0·3-ml aliquots of the filtrate were diluted to 3·0 ml with KRP buffer in the spectrophotometer cuvette. The amount of the 2-TP formed was determined at 343 mμ, as described.[1] In some experiments the tissues were heated at 90° for 2 min before addition of 2-PDS and incubation. In some cases, liver slices were treated with 70% ethanol (10 min at room temperature), then incubated with 10^{-3} M 2-PDS for 1 hr at 37° in a final solution of 50% ethanol. Duplicate determinations were carried out in all cases.

Oxygen uptake was measured in standard Warburg flasks as previously described.[5]

RESULTS AND DISCUSSION

When intact Ehrlich ascites cells are treated with 2-PDS, their respiration and glycolysis are strongly inhibited.[5] A reaction occurs between 2-PDS and components of the intact cells resulting in the formation of 2-TP. This is shown by the experiment recorded in Fig. 1. A constant amount of 2-PDS was incubated with increasing numbers of Ehrlich ascites cells, the amounts of 2-TP formed increased in a manner proportional to the number of Ehrlich ascites cells used. A point was eventually reached where all the 2-PDS had reacted and a further increase in the number of cells caused no further formation of 2-TP. The curve so obtained is similar to those obtained when increasing amounts of cysteine were used instead of Ehrlich ascites cells.[1]

Next, a fixed amount of Ehrlich ascites cells was treated with increasing amounts of 2-PDS, and the inhibitory effects of such treatment on oxygen uptake was determined.

It was found that the inhibition increases with the degree of reaction of the cells with 2-PDS (Fig. 2). Thus, the inhibition of the metabolism of Ehrlich ascites cells by 2-PDS is related to the reaction of this compound with cellular components. The nature of this interaction was further investigated.

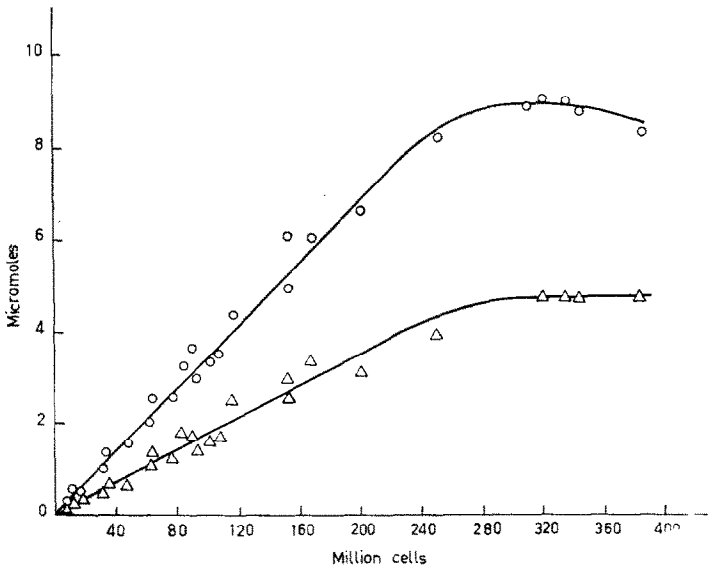


FIG. 1. Reaction of 2,2'-dithiodipyridine with increasing amounts of washed Ehrlich ascites cells. A solution of 2,2'-dithiodipyridine (4·74 μmole in Krebs–Ringer phosphate buffer, pH 7·2) was mixed with a suspension of the indicated number of Ehrlich ascites cells in the same buffer. The mixture (5·0 ml) was incubated for 1 hr at 37° in air, then centrifuged 10 min at 3,500 *g*. An aliquot (0·3 ml) of the supernatant solution was diluted to 3·0 ml with the same buffer. ○ = Formation of 2-thiopyridone, determined from the absorbance at 343 mμ; △ = disappearance of 2,2'-dithiodipyridine, determined from the absorbance at 233 mμ.

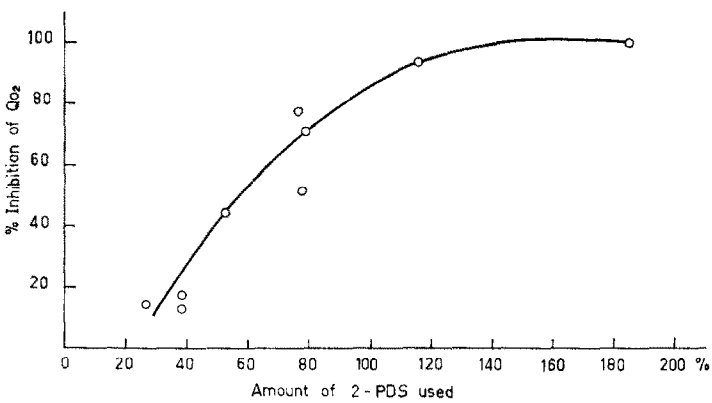


FIG. 2. Inhibition of oxygen uptake of washed Ehrlich ascites cells by 2 2'-dithiodipyridine. Each Warburg flask contained approximately 80 × 10^6 Ehrlich ascites cells (25 mg dry weight) and from 0–2 μmole of 2 2'-dithiodipyridine. The total volume per flask was 3·0 ml (pH 7·2, KRP buffer). Oxygen uptake was determined for 1 hr at 37° in air. The amounts of 2,2'-dithiodipyridine used are recorded as percentages of the maximum amount capable of reacting with Ehrlich ascites cells (4·75 m-mole of 2,2'-dithiodipyridine/100 g, dry wt.), corresponding to the formation of 9·49 m-mole of 2-thiopyridone/100 g, dry wt. (Table 1).

Incubation of intact washed Ehrlich ascites cells with an excess of 2-PDS resulted in the formation of greater amounts of 2-TP than when heat-denatured cells were used (Table 1). An even greater difference was found with mouse liver and kidney when

TABLE 1. REACTION OF EHRLICH ASCITES CELLS AND OF MOUSE TISSUES WITH 2,2′-DITHIODIPYRIDINE

Tissue preparation	Treatment	No. of expts.	2-TP (m-mole/100 g)* Range	Average ± S.E.
Ehrlich ascites cells	None	5	8·75–10·25	9·49 ± 0·24
Ehrlich ascites cells	Heat-denatured	5	7·46– 8·50	7·93 ± 0·20
Liver slices	None	11	3·82– 6·77	4·84 ± 0·28
Liver slices	Heat-denatured	5	2·06– 2·28	2·18 ± 0·04
Liver slices	Ethanol-denatured	3	2·23– 2·62	2·40 ± 0·10
Kidney slices	None	5	2·79– 3·39	3·07 ± 0·12
Kidney slices	Heat-denatured	5	1·27– 1·40	1·34 ± 0·02

* Dry weight for Ehrlich ascites cells, wet wt. for the other tissues.

interaction of 2-PDS with intact and heat-denatured slices was compared. Liver slices were also denatured by treatment with 70% ethanol, and the reaction with 2-PDS was carried out in 50% ethanol. The amount of 2-TP obtained under these conditions was within 10 per cent of that found with heat-denatured slices (Table 1). This result shows that the manner of denaturation does not affect the amount of reacting SH groups, and that the decrease in the amount of SH groups found with denatured slices is not due to trapping of 2-TP in the slices, since 2-TP is much more soluble in ethanol than in water.

A possible reason for this difference between live and denatured tissues may be an enzyme-mediated reaction, occurring in the intact cell, by which 2-PDS is reduced; such a reaction could not occur in the denatured tissues. Enzyme-catalyzed oxidation-reductions occurring in live cells are likely to involve the pyridine nucleotides. Reduced pyridine nucleotides could be oxidized by 2-PDS and in their turn cause an enzyme-catalyzed oxidation of other, nonsulfhydryl substrates. Direct evidence that 2-PDS can cause the oxidation of NADPH and of NADH was provided by the finding that 2-PDS is a substrate for gluthathione reductase, with either NADPH or NADH. This oxidation of NADPH (Reaction 2) can be coupled with the reduction of $NADP^+$ by glucose 6-phosphate, catalyzed by glucose 6-phosphate dehydrogenase (Reaction 3):

$$\text{2,2′-dithiodipyridine} + \text{NADPH} + \text{H}^+ \xrightarrow{\text{GR}} 2\ \text{(2-thiopyridone)} + \text{NADP}^+ \qquad (2)$$

$$\text{Glucose 6-phosphate} + \text{NADP}^+ \xrightarrow{\text{G6P-DH}} \text{6-Phosphogluconate} + \text{NADPH} + \text{H}^+ \qquad (3)$$

Summation of (2) and (3):

$$\text{2,2′-dithiodipyridine} + \text{glucose 6-phosphate} \rightarrow 2\ \text{(2-thiopyridone)} + \text{6-phosphogluconate}$$

The overall reaction is thus the oxidation of glucose 6-phosphate at the expense of 2-PDS. The course of this reaction is recorded in Fig. 3: after the NADPH present has reacted with 2-PDS (in excess) the reaction ceases; addition of glucose 6-phosphate dehydrogenase and glucose 6-phosphate causes the reaction to proceed until all the added glucose 6-phosphate is consumed; a further addition of glucose 6-phosphate initiates the reaction again.*

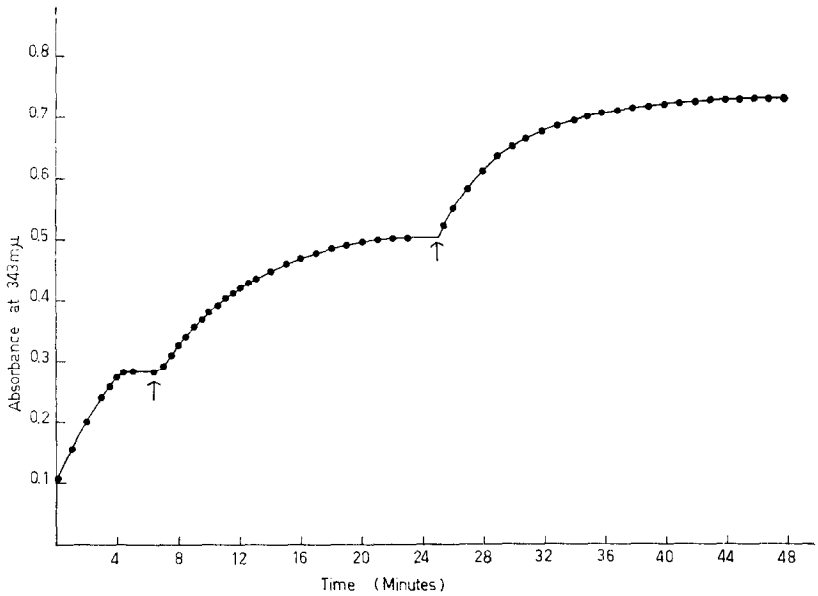


FIG. 3. Oxidation of glucose 6-phosphate in the presence of 2,2′-dithiodipyridine (Reactions 2 and 3 in text). The spectrophotometer cuvette contained 3·0 μmole of 2,2′-dithiodipyridine and 0·05 μmole NADPH in 3·00 ml of 0·1 M phosphate buffer, pH 7·5. Glutathione reductase (2·92 IU in 0·01 ml) was added to initiate the reaction. Glucose 6-phosphate dehydrogenase (1·82 IU in 0·01 ml) was added after 6 min. Aliquots of glucose 6-phosphate (0·05 μmole in 0·005 ml of 0·1 M phosphate buffer, pH 7·5) were added at 6·5 min and again at 25 min. A cuvette containing 3 μmole of 2-PDS in 3·00 ml of 0·1 M phosphate buffer, pH 7·5, was used as the blank.

The course of the oxidation of NADH by 2-PDS, catalyzed by GR (Reaction 4), is recorded in Fig. 4. The reaction ceases when the NADH present is consumed, and is initiated again by the addition of a further portion of NADH. Reaction 4 is

$$\text{2,2′-dithiodipyridine} + \text{NADH} + \text{H}^+ \xrightarrow{\text{GR}} 2\,(\text{2-thiopyridone}) + \text{NAD}^+ \qquad (4)$$

slower than the one with NADPH; it was found necessary to add a large amount of enzyme in order to obtain a convenient rate. Since two glutathione reductases have been reported, one NADPH-linked and the other NADH-linked,[6] it is possible that the enzyme preparations used in this work may have contained small amounts of the

* An interesting feature of Reactions 2 and 4 is that for each mole of NADPH or NADH consumed, 2 mole of 2-TP are formed. The spectral properties of the reduced pyridine nucleotides (λ_{max} = 340 mμ, E = 6·22 × 10³) and of 2-TP (λ_{max} = 343 mμ, E = 7·06 × 10³) are quite similar. The change in absorbance at 343 mμ during the course of these two reactions reflects the combined change due to disappearance of 1 mole of NADPH (or NADH) and formation of 2 mole of 2-TP. The change in absorbance is thus a little more sensitive than the simple disappearance of NADPH or NADH.

NADH-dependent enzyme, this impurity being the cause of the observed slow Reaction 4.

2-PDS is capable, as we have seen, of causing the oxidation of compounds such as NADPH and NADH through reactions involving GR (Reactions 2 and 4). 2-PDS is

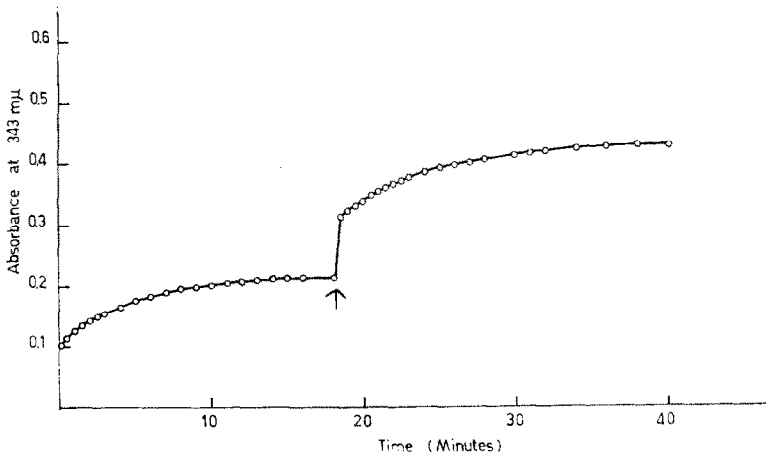


FIG. 4. Oxidation of NADH by 2,2′-dithiodipyridine and glutathione reductase (Reaction 4 in text). The spectrophotometer cuvette contained 3·0 μmole of 2-PDS and 0·05 μmole of NADH in 2·80 ml of 0·1 M phosphate buffer, pH 7·5. Glutathione reductase (20 IU in 0·20 ml) was added to initiate the reaction. NADH (0·05 μmole in 0·05 ml of the same buffer) was added after 18 min. The blank cuvette contained 3·0 μmole of 2,2′-dithiodipyridine in 3·00 ml of 0·1 M buffer.

itself a substrate for this enzyme, reacting at approximately 1/200 the rate of GSSG with NADPH; with NADH the rate was found to be about 1/800 that of GSSG. Direct nonenzymatic reaction between 2-PDS and NADH or NADPH does not occur at pH 7·2. The very rapid nonenzymatic reaction of 2-PDS with thiols (including GSH, coenzyme A, lipoic acid, and cysteine)[1] to give the corresponding disulfides, as indicated in Reaction 1, can lead to an accelerated oxidation of nonsulfhydryl cell components by 2-PDS. This is exemplified in Reactions 5 and 6 for NADPH:

$$\text{GSSG} + \text{NADPH} + \text{H}^+ \xrightarrow{\text{GR}} 2\ \text{GSH} + \text{NADP}^+ \tag{5}$$

$$\text{2,2′-dithiodipyridine} + 2\ \text{GSH} \longrightarrow 2\ \text{(2-thiopyridone)} + \text{GSSG} \tag{6}$$

That such a system is actually capable of operating is shown in Fig. 5. During the initial reaction of GSSG with NADPH in presence of GR, the absorbance at 343 mμ decreases due to the oxidation of NADPH. When 2-PDS is added, the absorbance increases due to formation of 2-TP; the GSSG formed reacts with NADPH, then the reaction stops. Further additions of 2-PDS repeat the process, until all the NADPH present is consumed. At this point the addition of more 2-PDS does not cause a further increase in the absorbance. This reaction causes the oxidation of NADPH to proceed at a much faster rate than Reaction 2, and may be responsible for a substantial amount of the oxidation of nonsulfhydryl compounds within cells by 2-PDS.

The central position of glutathione in cell metabolism[7–9] permits one to speculate that the continued reoxidation of available intracellular GSH caused by 2-PDS could lead to the oxidation of many other substrates and cofactors. This catalytic process may continue until a majority of the enzymatically oxidizable, pyridine nucleotide-linked substrates within the cell are in the oxidized state. Materials escaping oxidation

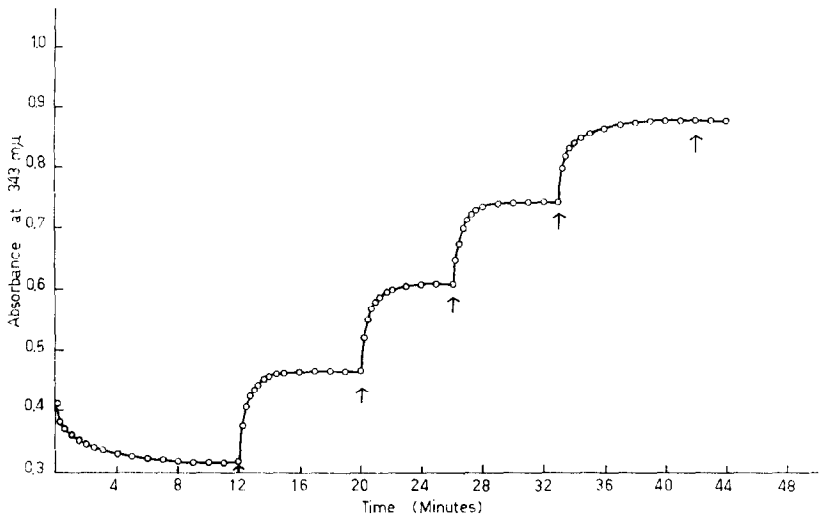


FIG. 5. Effect of 2,2′-dithiodipyridine on the oxidation of NADPH by glutathione (Reactions 5 and 6 in text). The spectrophotometer cuvette contained 0·05 μmole of oxidized glutathione and 0·2 μmole of NADPH in 3·00 ml of 0·1 M phosphate buffer, pH 7·5. Glutathione reductase (0·5 IU in 0·005 ml) was added to initiate the reaction. Aliquots of 2,2′-dithiodipyridine (0·05 μmole in 0·05 ml of buffer) were added at the times indicated by the arrows. The blank was 0·1 M phosphate buffer, pH 7·5.

would be: (a) substrates not directly oxidizable by 2-PDS, if the enzymes mediating their oxidation are inhibited by 2-PDS; and (b) sulfhydryl groups masked by protein folds and thereby inaccessible to 2-PDS.

A study of the properties of 2-PDS-inhibited cells, as well as experiments attempting to reverse the inhibition, are currently in progress. Further uses of 2-PDS as an analytical tool are also being developed.

Acknowledgements—The authors wish to thank Dr. S. Abraham for helpful discussions and advice, and Dr. M. E. Brokke for the preparation of 2-PDS.

REFERENCES

1. D. R. GRASSETTI and J. F. MURRAY, JR., *Archs. Biochem. Biophys.* **119**, 41 (1967).
2. W. W. UMBREIT, R. H. BURRIS and J. F. STAUFFER, *Manometric Techniques*, p. 149. Burgess, Minneapolis (1957).
3. W. MARCKWALD, W. KLEMM and H. TRABERT, *Ber.* **33**, 1556 (1900).
4. H. MCILWAIN and H. L. BUDDLE, *Biochem. J.* **53**, 412 (1953).
5. D. R. GRASSETTI, M. E. BROKKE and J. F. MURRAY, JR., *J. mednl. Chem.* **8**, 753 (1965).
6. H. D. HORN, in *Methods of Enzymatic Analysis* (Ed. H. U. BERGMEYER), p. 875. Academic Press, New York (1963).
7. P. C. JOCELYN, in *Glutathione* (Ed. E. M. CROOK), p. 43. Cambridge University Press, Cambridge (1959).
8. S. RAPOPORT and D. SCHEUCH, *Acta biol. med. germ. suppl.* 3, 203 (1964).
9. H. S. JACOB and J. H. JANDL, *J. biol. Chem.* **241**, 4243 (1966).